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14. ABSTRACT

The discovery of a single nucleotide polymorphism (SNP) in the mdm2 promoter uncovered a previously unknown role of this SNP in predicting early onset of breast and the possibility that this germ line variation could decrease the effectiveness of treatment. These outcomes are likely due to the increased expression of mdm2 protein in SNP309 individuals, which blunts the p53-mediated apoptotic response to DNA damage. The objective of this proposal is to test the hypothesis that SNP309 decreases the effectiveness of radiation and chemotherapy in breast cancer and that this negative impact can be overcome by targeted down-regulation of mdm2. There appears to be a trend toward excess contralateral events with the variant and enrichment of the variant in ER+ breast cancer recurrences. We observed that anti-estrogen agent, fulvestrant, causes a decrease in mdm2 protein half-life, leading to a reduction in mdm2 following treatment with this agent. We demonstrate that combined use of fulvestrant with chemotherapeutic drugs doxorubicin, etoposide and paclitaxel can enhance the sensitivity of breast cancer cells to these cytotoxic agents. We observed that mdm2 expression is differentially modulated by estrogen, the anti-estrogen tamoxifen, and genistein in a genotype-specific manner. The largest effects on reduction in mdm2 expression at the protein level occur in the mdm2 SNP309 cell line. We will continue to explore mechanistic studies in vitro while evaluating the clinical outcome associations of SNP309 to chemotherapy, hormonal therapy and radiation therapy.

15. SUBJECT TERMS
mdm2, breast cancer, polymorphisms

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INTRODUCTION

The recent discovery of a single nucleotide polymorphism (SNP) in the mdm2 promoter uncovered a previously unknown role of this SNP in predicting early onset of breast and the possibility that this germ line variation could decrease the effectiveness of treatment. These outcomes are likely due to the increased expression of mdm2 protein in SNP309 individuals, which blunts the p53-mediated apoptotic response to DNA damage. The objective of this proposal is to test the hypothesis that SNP309 decreases the effectiveness of radiation and chemotherapy in breast cancer patients and that this negative impact can be overcome by targeted down-regulation of mdm2. The rationale in support of these objectives are molecular epidemiological data showing that individuals harboring SNP309 are at increased risk for early onset breast cancer, and laboratory studies showing that SNP309 decreases the activity of DNA damaging agents. If we are to achieve better results of treatment for patients with breast cancer, the choice of treatment must eventually benefit from a more precise understanding of the genetic abnormalities that are present in each individual's tumor. Using the same dose of drug or amount of radiation for each breast cancer patient cannot possibly be consistent with our understanding of modern molecular medicine. For example, subtle variations in our genetic code (called single nucleotide polymorphisms, [SNPs "snips"]) exist in the human population and make us susceptible to certain diseases and resistant to others. Similarly, these polymorphisms can make us more or less sensitive to treatment. Since these polymorphisms exist both in breast cancer and in normal tissues, understanding their impact on both the patient and the tumor will eventually guide the choice and dose of drug and amount of irradiation. Therefore, our objective is to improve the ways in which patients with breast cancer are evaluated and treated through an understanding of subtle variations in the human genome. The proposal brings together a team of molecular biologists/epidemiologists, pharmacologists, radiation and medical oncologists, and statisticians to focus on this novel approach to breast cancer treatment.

BODY

Task 1. Determine the impact of mdm2 SNP309 on the results of breast irradiation

Updating and assuring complete clinical data has been ongoing. Paperwork for IRB in accordance with recommendations from the IRB at CINJ and the human investigations committees of the DOD was completed and IRB-approval obtained. Patient accrual was initiated through the Radiation Oncology Clinics.

We have completed analysis of mdm2 on the cohort of patients whom we have long term follow-up. We confirmed an association of SNP309 with young patient age in the population of over 250 patients previously treated with long-term follow-up. While all patients in the previously treated database were in the younger age group, a larger percentage of patients of the GG genotype were under age 40 compared to the TT/TG genotypes (65% vs 35%, $p < .01$). We also found a correlation with race, with few

African American patients having the GG homozygous genotype at SNP309. There were no other strong correlations between the SNP309 status and clinical-pathologic variables such as histology, ER status, Her2 status, nodal status, T-stage, family history. There did not appear to be strong correlations with local-regional outcome in this dataset. There appears to be a trend toward excess contralateral events with a 10-year event rate of 9% in the TT/TG subset compared to over 20% in the GG. This will be analyzed in the larger prospective data set in the future. In addition, in this data set there was a difference in distant metastasis in the GG subtypes, with the 10 year rate of distant metastasis-free survival 89% in the TT/TG subset compared to 76% in the GG subtype ($p = .04$). This will be further explored in multivariate analysis. Although there were no clear differences in local control, further exploratory subset analysis will be performed to determine if there are subsets within this cohort with higher local relapse rates.

In the prospective cohort, we continue to recruit patients in the radiation therapy clinic as well as in CINJ breast clinic. In the radiation therapy clinic we continue to actively accrue patients and continue accrual in the CINJ clinics such that we will have reached our accrual goals of patients treated with breast conserving surgery and radiation by years end. We will then analyze this larger cohort for SNP309 and evaluate outcomes and clinical-pathologic correlations over the next year.

Task 2 Determine the impact of mdm2 SNP309 on the results of adjuvant chemotherapy.

A total of 2453 women have been consented for participation in the parent study protocol as of May 12, 2010 (CINJ Protocol #040406, IRB# 0220044862). Of these, genomic DNA has been isolated from 1,720 patients. The information contained in Table 1 reflects data available from chart review for study participants (this chart review was completed as of February 15, 2010).

Table 1- Demographics of Study Cohort at The Cancer Institute of New Jersey.

Race	Number of Patients	% of Patients
African American	57	5.7
Asian	41	4.1
Caucasian	771	77.3
Hispanic	61	6.1
Indian	25	2.5
Other	43	4.3

Tumor Type	Number of Patients	% of Patients
Colloid/Mucinous	12	1.3
DCIS	93	9.8
Invasive Ductal	705	74.5
Invasive Lobular	94	9.9
Medullary	6	0.6
Metaplastic	4	0.4
Other	32	3.4
Unknown	52	n/a

ER Status	Number of Patients	% of Patients
Positive	748	74.9
Negative	250	25.1

PR Status	Number of Patients	% of Patients
Positive	638	63.9
Negative	361	36.2

Her2/Neu Status	Number of Patients	% of Patients
Not amplified or 0-2+ IHC	553	79.9
Amplified or 3+ IHC	145	20.1
(all 2+ by IHC were reflexed for FISH)		

Stage	Number of Patients	% of patients
0	93	9.3
1	345	34.6
IIA	198	12.5
IIB	125	12.5
IIIA	70	7.0
IIIB	25	2.5
IIIC	20	2.0
IV	40	4
Unknown	156	15.6

Tumor	% of Patients
T0	5.2
T1	46.3
T2	24.7
T3	6.5
T4	4.6

Node status	
N0	47.6
N1	32.5
N2	3.7
N3	0.2

Metastatic Status	
M0	86
M1	4

Recurrence Status	% of patients
Yes	20.3
No	79.7
(excludes stage IV at diagnosis)	

The timing of recurrence is an important variable in this dataset since the median follow-up time is 7.2 years. Of 160 recurrences, however, 71% occur by the end of 5 years (Table 2). The majority of recurrences beyond five years reflect estrogen receptor positive disease.

Table 2. Time to recurrence of breast cancer from date of initial biopsy-proved disease.		
Year(s) to recurrence	n	% of all recurrences
1	13	0.081
2	39	0.243
3	23	0.144
4	19	0.119
5	21	0.131
6	10	0.063
7	5	0.031
8-10	8	0.050
>10	22	0.138

The nature of recurrence reflects the initial stage, molecular features, and type of therapy given adjuvantly. Table 3 depicts the distribution of adjuvant therapies delivered in this cohort of breast cancer patients. The majority of patients received radiation, chemotherapy, and/or hormonal therapy. Only about 12% of patients received trastuzumab.

Table 3. Distribution of the adjuvant therapy received by breast cancer patients in this cohort.		
Patients Receiving Each Treatment	No (%)	Yes (%)
Radiation	22.8	77.2
Chemotherapy	32.7	67.3
Hormonal therapy	27.7	72.3
Trastuzumab	87.7	12.3

We will be using this cohort to determine the genotype-specific recurrence free survival for the following: 1) hormone receptor positive and hormone receptor negative breast cancers; 2) hormone receptor positive breast cancer patients receiving hormonal therapy alone; 2) breast cancer patients receiving chemotherapy only (hormone receptor positive and negative disease); 3) breast cancer patients receiving chemotherapy followed by hormonal therapy (hormone receptor positive only).

Breast Cancer Recurrence as a Function of Receptor Status, MDM2 SNP309 Genotype, and Adjuvant Therapy

Of 157 recurrences with known genotypes, more than 50% were in estrogen receptor negative (ER-) breast cancers, as expected. In estrogen receptor negative breast cancer, the recurrence rate was 29% as compared to 16% in estrogen receptor positive (ER+) disease. There is no significant difference in risk of recurrence by genotype for either estrogen receptor positive or estrogen receptor negative breast cancers (Table 4). For ER- disease risk of GG vs. TT genotype, OR 1.132 CI [0.594-2.158], p=0.707. For ER+ disease, OR for recurrence for GG as compared with TT was 1.329 CI [0.837-2.11],

p=0.227. Although the frequency of recurrence for GG ER- is 20% and for ER+ is 13%, this is not statistically significant (p=0.41).

Table 4. Rates of breast cancer recurrence as a function of hormone receptor status and use of adjuvant hormone therapy.

	ER-/no hormone therapy		ER+/hormone therapy	
	No recurrence	Recurrence	No Recurrence	Recurrence
TT	50 (0.35)	19 (0.32)	190 (0.38)	31 (0.32)
TG	70 (0.49)	28 (0.47)	224 (0.45)	54 (0.55)
GG	23 (0.16)	12 (0.20)	85 (0.17)	13 (0.13)

Because of the lack of targeted therapy for hormone receptor negative disease, its more aggressive behavior and propensity to recur, the majority of patients with hormone receptor negative disease received chemotherapy (Tables 5, 6). Those patients with ER- disease receiving chemotherapy demonstrate an enrichment of GG genotype in those that recur as compared to other genotypes. This was not significant: OR 1.566 CI [0.608-4.036], p=0.352. However, in ER+ patients receiving chemotherapy, heterozygotes are enriched in those recurring but this did not reach statistical significance.

Table 5. Rates of ER- breast cancer recurrence by MDM2 SNP309 genotype in those receiving adjuvant chemotherapy.

	ER-/no chemo		ER-/chemo	
	No recurrence	Recurrence	No Recurrence	Recurrence
TT	8	1	41 (0.36)	16 (0.30)
TG	2	0	54 (0.48)	26 (0.49)
GG	0	0	18 (0.16)	11 (0.21)

Table 6. Rates of ER+ breast cancer recurrence by MDM2 SNP309 genotype in those receiving adjuvant chemotherapy.

	ER+/no chemo		ER+/chemo	
	No recurrence	Recurrence	No Recurrence	Recurrence
TT	70 (0.38)	7	88 (0.37)	20 (0.29)
TG	84 (0.46)	5	106 (0.45)	40 (0.58)
GG	30 (0.16)	3	44 (0.18)	9 (0.13)

Association of MDM2 SNP309 with Recurrence of Early Stage Breast Cancer

Because stage III disease has the highest risk of recurrence due to its advanced nature, early stage disease was then analyzed separately. This included stage 0 through stage IIB disease. Again, there is an insignificant enrichment of the heterozygotes recurring in those that were ER+ and received hormone therapy. Overall, recurrence rates were similar between ER- disease and ER+ disease by genotype (Table 7). This finding is significant because hormone receptor positive disease has a better prognosis than hormone receptor negative disease in general.

Table 7. Rate of breast cancer recurrence in ER+ and ER- disease by MDM2 SNP309 genotype and use of adjuvant hormone therapy.				
	ER-/no hormone therapy		ER+/hormone therapy	
	No recurrence	Recurrence	No Recurrence	Recurrence
TT	25 (0.31)	11 (0.37)	116 (0.36)	20 (0.29)
TG	42 (0.53)	15 (0.50)	148 (0.46)	37 (0.54)
GG	13 (0.16)	4 (0.13)	58 (0.18)	11 (0.16)

Site-Specific Recurrence as a Function of MDM2 SNP309

We analyzed the site of recurrence for stages 0-III breast cancer as a function of MDM2 genotype. There were few cases where recurrences were regional or multiple sites including local, regional, and distant loci (n=11). Therefore, most recurrences were either local (n=49) or distant only (n=49). While genotype did not associate with risk of local recurrence, G carriers had a higher risk of recurrence: OR 2.188 CI [1.070-4.477], p=0.028. Pattern of recurrence in G carriers also favored distant over local recurrences: OR 3.263 CI [1.262-2.456], p=0.013. The lack of association with local recurrence rate, but association with distant recurrence confirms the finding in Aim 1.

Combinatorial Analysis of MDM2 SNP309 with MDM4 Genotypes

Because we had previously shown that the variant G allele of MDM2 SNP309 associates with earlier age of diagnosis of ductal breast cancers (1) and more recently demonstrated in the same population that the variant T allele of MDM4 also results in earlier age of diagnosis of ductal breast cancers (2), we asked whether the combination of each risk allele would further modify the age at diagnosis of ductal breast cancers. The combination of the risk genotypes of MDM4 with MDM2 results in the earliest onset of estrogen receptor negative breast cancer. The mean age of diagnosis for MDM4/MDM2 combinations were 41.9 and 50.8 for TT/TG and CC/TG, respectively (Δ=8.9 years; p=0.0099). There were insufficient numbers to compare homozygous variants for both MDM4 and MDM2 with the combination wildtype. There was only one TT/GG combination, diagnosed at age 42. In contrast, in estrogen receptor positive breast cancer, the MDM4 risk allele appears to negate the previously-observed earlier onset of the MDM2 SNP309 G allele. For example, when MDM4 was homozygous wildtype, there was a 1.8 year difference in age of onset where the GG combination was diagnosed earlier. When the MDM4 homozygous variant TT was combined with MDM2 SNP309, the age of diagnosis was 54.2 years and 51.9 years for the TT/TT and TT/TG combined genotypes. Although the combined TT/GG variants showed an age of diagnosis of 64 years, there were only 3 cases, underpowering this comparison.

Task 3 Determine the ability of anti-estrogens to restore drug and irradiation sensitivity by decreasing mdm2 expression

In this grant period, we have investigated the effects of anti-estrogen agent, fulvestrant, on mdm2 expression and sensitivity of human breast cancer cells to chemotherapeutic drugs. We found that in both MCF7 (T/G) and T47D (G/G) human breast cancer cell lines, fulvestrant decreases mdm2 expression to similar extents (Figure 1). Further, fulvestrant not only abolished the effect of estradiol (E_2), but also was also able to suppress mdm2 protein levels below the control (no E_2) level (Figure 2). Mdm2

depletion by fulvestrant did not correlate with an increase in p53 activation (slight decrease) and no change in p21 levels was observed (Figure 3). Fulvestrant did not cause a reduction in mdm2 mRNA, but reduces mdm2 protein half-life (Figure 4). The combination of fulvestrant and chemotherapeutic drugs doxorubicin, etoposide or paclitaxel showed synergism in MCF7 and T47D cells (Figure 5).

Epidemiologic evidence suggests that genistein intake is inversely related to the risk of several tumors including breast cancer but its mechanism of action is not completely understood. However, conflicting data exists on the effect of genistein on the expression of the estrogen-dependent mdm2 gene. We hypothesized that if genistein acted like an anti-estrogen, it could bind estrogen receptor (ER), preventing binding to the ERE at the mdm2 promoter and lead to down-regulation of mdm2 expression. For those cells in which SNP309 is present, we anticipated even stronger effects. To explore this, we grew breast cancer cells under conditions of no estrogen (PF), normal media (N), with estradiol (E2), with Tamoxifen (T), and with genistein (G). We selected three ER+ breast cancer cell lines representing the three mdm2 SNP309 genotypes: ZR75-1 (TT), MCF-7 (TG), and T47D (GG). Protein was isolated from the cells grown in the various conditions and Western blot analysis was performed (Figure 6).

In MCF-7 cells (TG), mdm2 protein is reduced when cells are grown in the absence of estrogen media as compared with normal media or with estradiol. With Tamoxifen or genistein, relative to estradiol, mdm2 was reduced, but remained at levels higher than that in the absence of estrogen. In T47D (GG genotype), the response in the absence of estrogen, normal media, and with estradiol treatment is similar to that of MCF-7 cells (TG genotype). However, by comparison, mdm2 levels are reduced to levels nearly equivalent to those in the absence of estrogen when treated with Tamoxifen and genistein. Of interest, the ~50kDa isoform of mdm2 is reduced further with genistein as compared with Tamoxifen, suggesting an effect on alternative splicing. In ZR75-1 cells (TT), no 50kDa isoform is expressed. In contrast to the MCF7 and T47D cells, genistein and Tamoxifen treatment resulted in ***increased*** mdm2. Increased expression may be the result of increased transcription or posttranslational changes leading to reduced degradation and longer half-life. These results suggest a genotype-specific effect of genistein and may explain contradictory effects observed in studies.

The P2 promoter of mdm2 has an ERE and we previously demonstrated that mdm2 levels are estradiol dose-dependent and genotype dependent (preliminary data for proposal). Therefore, we had hypothesized that Tamoxifen, an anti-estrogen that binds ER, would result in decreased mdm2 as well as decreased binding at the promoter as determined by chromatin immunoprecipitation (figure 7). While this was true in ZR75-1 cells and to a much lesser degree in MCF7 cells, binding occurred in the presence of Tamoxifen in T47D. As genistein is thought of as an anti-estrogen, we hypothesized that genistein treatment would result in decreased binding to the ERE. With genistein treatment, ER still bound the P2 promoter region but transcription was reduced in MCF7 and T47D. Interestingly, binding appeared to be reduced in ZR75-1 for treatment with estradiol, Tamoxifen, and genistein. Since protein levels were increased in ZR75-1 with Tamoxifen and genistein, this suggests that post-translational modification leading to longer half-life

may play a role in increased mdm2 levels with these treatments. It is not clear if this is truly a genotype-specific effect or if this is related to this particular cell line.

KEY RESEARCH ACCOMPLISHMENTS

- We observed that anti-estrogen agent, fulvestrant, causes a decrease in mdm2 protein half-life, leading to a reduction in mdm2 following treatment with this agent.

- We demonstrate that combined use of fulvestrant with chemotherapeutic drugs doxorubicin, etoposide and paclitaxel can enhance the sensitivity of breast cancer cells to these cytotoxic agents.

- We observed that mdm2 expression is differentially modulated by estrogen, the anti-estrogen tamoxifen, and genistein in a genotype-specific manner. The largest effects on reduction in mdm2 expression at the protein level occur in the mdm2 SNP309 cell line.

- We observed that binding of estrogen receptor alpha to the mdm2 promoter is less efficient in the wildtype mdm2 breast cell line in the presence of estrogen, tamoxifen, and genistein as compared with cell lines carrying at least one variant allele.

- We have accrued the patients needed to evaluate the role of SNP309 in mdm2 on outcomes associated with chemotherapy and hormonal therapy.

- We have analyzed associations between MDM2 SNP309 and breast cancer phenotypes.

REPORTABLE OUTCOMES

Manuscript

None

Abstracts

Era of Hope poster

Degree obtained that are supported by this award

None

CONCLUSIONS

1. Selective estrogen receptor down-regulator, fulvestrant, decreases MDM2 expression and enhances sensitivity of human breast carcinoma cells to chemotherapeutic drugs (such as doxorubicin, etoposide and paclitaxel).
2. The anti-estrogen tamoxifen decreases MDM2 expression in a genotype-specific manner.
3. MDM2 SNP309 G allele associates with increased risk of distant recurrence of breast cancer.
4. MDM2 SNP309 G allele associates with increased risk of contralateral breast cancer events.

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2. Kulkarni DA, Vazquez A, Haffty BG, Bandera EV, Hu W, Sun YY, Toppmeyer DL, Levine AJ, Hirshfield KM. A polymorphic variant in human MDM4 associates with accelerated age of onset of estrogen receptor negative breast cancer. Carcinogenesis 2009, 30: 1910-5.

APPENDICES: none

SUPPORTING DATA

Figure 1

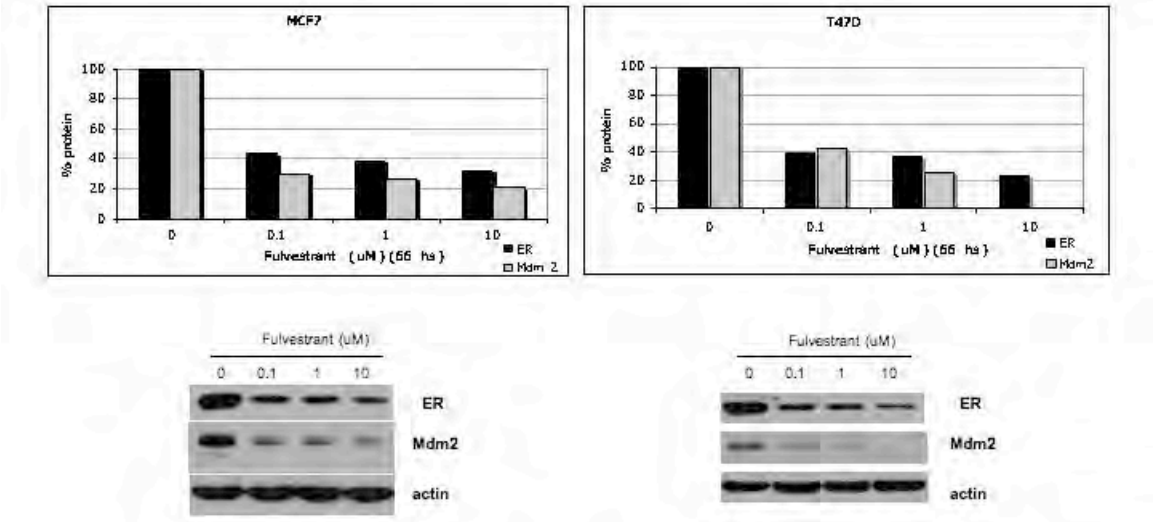

Figure 1. Effect of the antiestrogen fulvestrant on expression of estrogen receptor and mdm2 proteins. Two breast cancer cell lines MCF7 and T47D were grown at various

concentrations (0-10 micromolar) of fulvestrant for 66 hours. Protein was then harvested and levels of estrogen receptor and mdm2 were assayed by Western blot. The upper plots demonstrate the dose-dependent reduction of both proteins in each cell line.

Figure 2

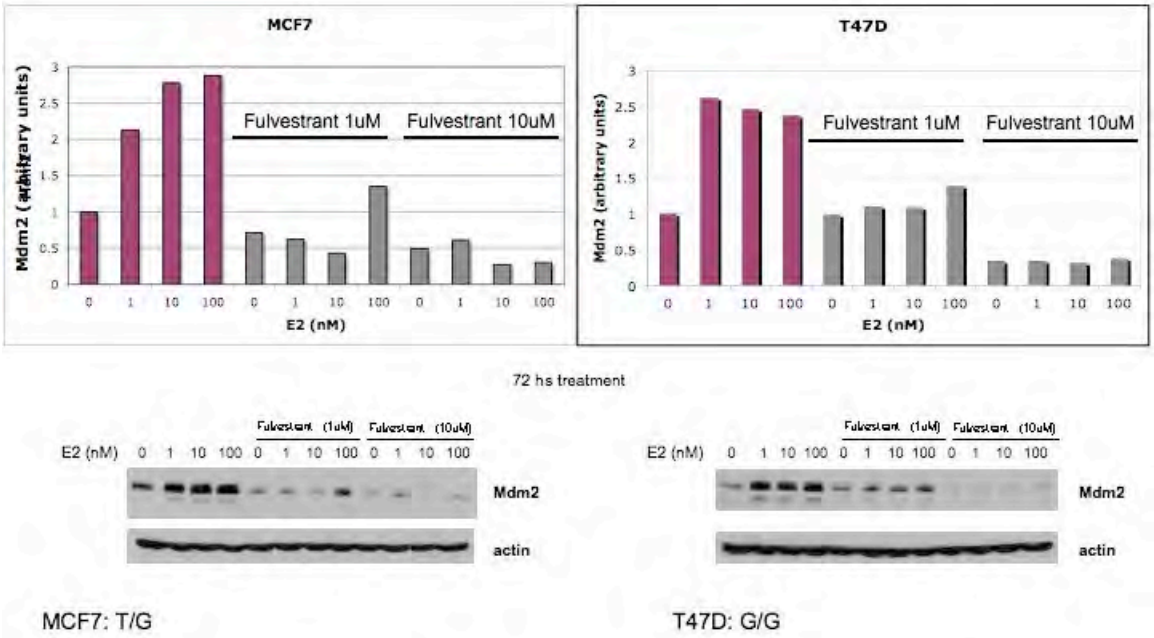

Figure 2. Effect of the antiestrogen fulvestrant on mdm2 levels in breast cancer cells grown in the presence of estradiol. Two breast cancer cell lines MCF7 and T47D were grown in the presence of estradiol, and estradiol with one of two concentrations of fulvestrant. Thee lower plots represent the Western blot analysis corresponding to the quantification in the upper graphs.

Figure 3

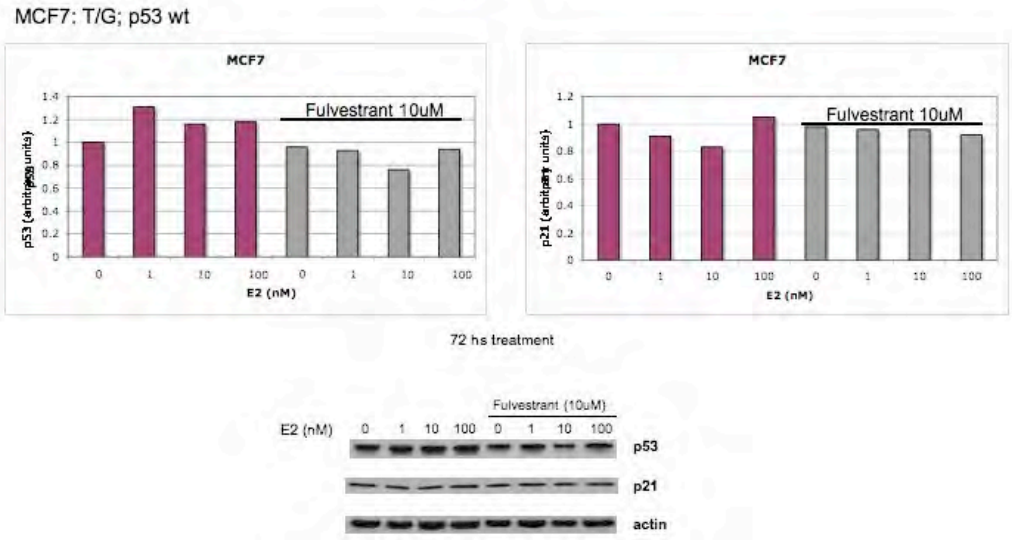

Figure 3. Effect of estradiol and the antiestrogen fulvestrant on p53 and p21 in breast cancer cell lines. The breast cancer cell lines MCF7 was grown in estradiol alone or with the presence of 10micromolar fulvestrant. Protein was harvested and Western blot analysis performed to detect p53 and p21. The lower plot depicts the Western blot for each protein using actin as a loading control. This plot was used to quantitate protein levels expressed in the upper curves.

Figure 4

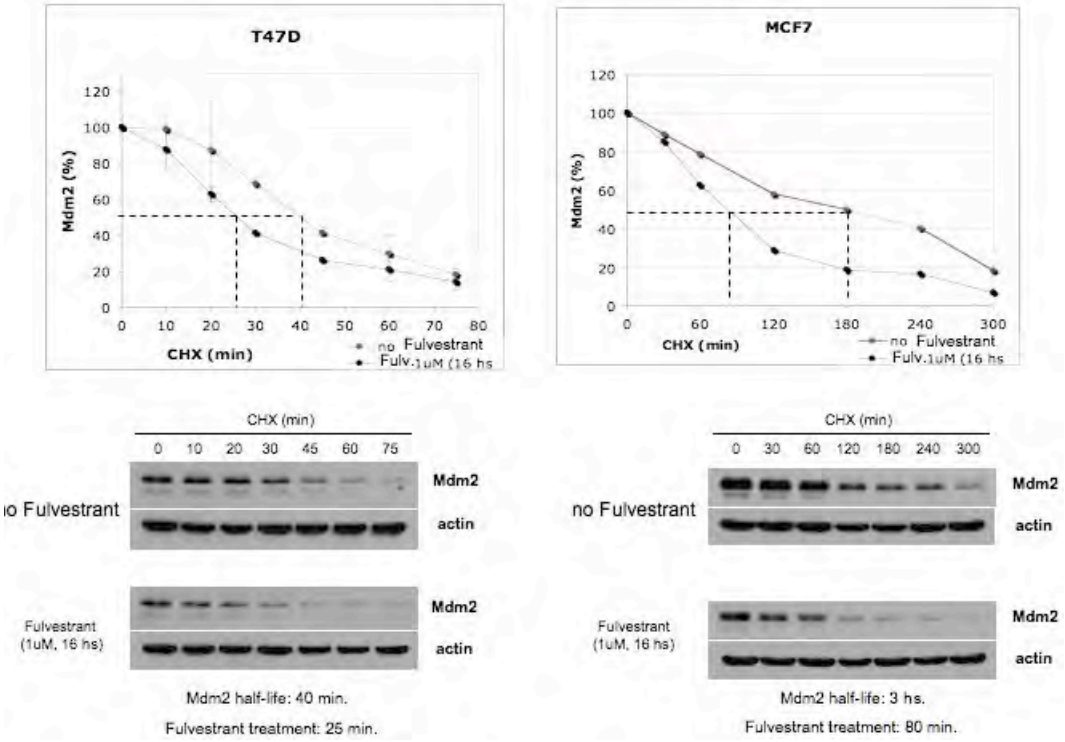

Figure 4. Effect of fulvestrant on the half-life of mdm2 protein. Two breast cancer cell lines T47D and MCF7 were grown in the absence and the presence of the antiestrogen fulvestrant. Cell were treated with cycloheximide (CHX) and mdm2 protein expression was determined at various time points. The lower curves show Western Blot analyses from each cell type using actin as a loading control and were used to quantitate mdm2 levels given in the corresponding curves above.

Figure 5

			CompuSyn Analysis:
MCF7	Fulvestrant : Doxorubicin at constant ratio	1 : 0.5	Synergism
		1 : 0.015	Synergism
	Fulvestrant : Paclitaxel at constant ratio	1 : 0.025	Synergism / Additive
		1 : 0.0005	Synergism
	Fulvestrant : Etoposide at constant ratio	1 : 1	Synergism
		1 : 5	Synergism
T47D	Fulvestrant : Doxorubicin at constant ratio	1 : 0.25	Synergism / Additive
		1 : 0.0035	Synergism / Additive
	Fulvestrant : Paclitaxel at constant ratio	1 : 0.0125	Synergism / Antagonism
		1 : 0.0002	Synergism / Antagonism
	Fulvestrant : Etoposide at constant ratio	1 : 10	Synergism / Additive
		1 : 0.07	Synergism / Additive / Antagonism

Figure 5. Effect of combining the antiestrogen fulvestrant with doxorubicin, paclitaxel, or etoposide in two breast cancer cell lines. Analysis of cell response was determined using the CompuSyn program. Each combination was observed at two concentrations of chemotherapeutic agent while keeping the concentration of fulvestrant constant. The last column indicates the type observed effect of the combination for each drug and dose.

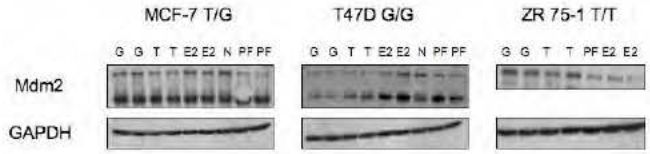

Figure 6. Western blot demonstrates mdm2 protein expression in three ER+ breast cancer cells lines representing the three SNP309 genotypes: ZR75-1 (TT), T47D (GG), MCF7 (TG). Cells were grown under different conditions: phenol-free, charcoal stripped media (PF), normal media (N), estradiol (E2), Tamoxifen (T), or genistein (G).

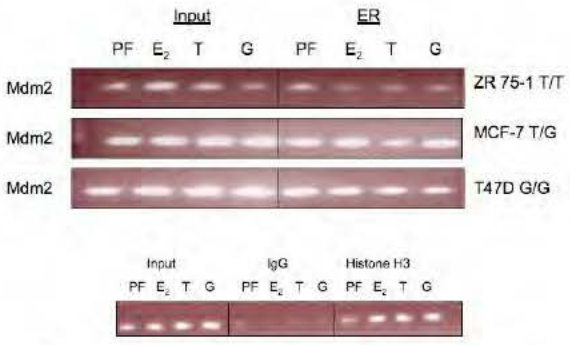

Figure 7. Chromatin immunoprecipitation using anti-ERalpha antibody with PCR of the mdm2 P2 promoter region was performed in the three ER+ breast cancer cell lines representing each of the three mdm2 genotypes.